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㉘ SnRNP-A antigen and fragments thereof.

㉙ The present invention relates to a protein antigen which is reactive towards an auto-antibody which is associated with an auto-immune disease, and the use of this protein in diagnostic tests relating to auto-immune diseases.

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## snRNP-A antigen and fragments thereof.

The invention relates to a protein antigen which is reactive towards an auto-antibody which is associated with an auto-immune disease, and the use of this protein in diagnostic tests relating to auto-immune diseases.

5 In healthy humans and animals, after the intrusion of a foreign substance (the antigen) the body will attempt to attack this antigen by generating specific antibodies which are directed against this antigen. Most individuals are in general tolerant to substances which occur in their own body. Some individuals on the other hand generate antibodies against endogenous substances, tissues, or components. Such antibodies (auto-antibodies) cause great damage to the organs which contain these endogenous substances. The  
10 development of the associated auto-immune disease is in general very slow (a matter of years) and this hampers timely clinical diagnosis and treatment to a high degree. Diagnosis can generally only be made after appreciable damage has already been caused to the body. Earlier research has shown that many of these auto-antibodies are syndrome-specific, i.e. the disease seems to be characterized by the occurrence of specific auto-antibodies.

15 Furthermore, it appears from recent research that these specific auto-antibodies can often be detected in the serum of a patient long before the clinical diagnosis can be made with certainty. The auto-antibodies therefore predict, as it were, which disease is developing.

The timely detection of these auto-antibodies in the patient's serum is the more important because the patient's treatment can then be initiated earlier, thereby delaying, or even preventing, the often serious  
20 damage during the later phase of the disease.

Patients with an auto-immune disease possess auto-antibodies directed against one or more protein antigens, such as small protein molecules which occur in the cell nucleus and which are complexed with ribonucleic acid, such as the so-called snRNPs (small nuclear ribonucleoproteins).

A snRNP protein and fragments thereof having a well defined amino acid sequence have now been  
25 found, which render it possible to diagnose immune diseases, especially systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD), at an early stage.

The invention therefore relates to a protein with the structure as shown in formula 1 which protein is substantially free from other natural material, and to fragments thereof which are immunochemically reactive, and to peptides which contain such fragments.

30 The invention furthermore relates to preparative methods for such a protein, fragment or peptide, to diagnostic methods for the detection of auto-immune antibodies with the aid of such a protein, fragment, or peptide and to test kits for carrying out such diagnostic methods, and to pharmaceutical compositions for combating auto-immune diseases, especially SLE and MCTD, which compositions contain such a protein, fragment or peptide.

35 The invention furthermore relates to DNA which codes for the protein, the fragments and the peptides according to the invention, to a vector which contains the DNA, and to a host which contains such a vector.

The protein according to the invention is known in the literature as the U<sub>1</sub>-snRNP-A protein. This protein, however, has been insufficiently purified in the literature. Structure elucidation has been impossible hitherto.

40 By using a DNA clone which contains the nucleotide sequence coding for human U<sub>1</sub>-snRNP-A protein, and which clone was obtained using recombinant DNA techniques known per se, it was possible to elucidate, by techniques known per se, the amino acid sequence of the U<sub>1</sub>-snRNP-A protein, as is shown in formula 1.

Some fragments of the protein according to the invention react specifically with auto-antibodies against  
45 the U<sub>1</sub>-snRNP-A protein. Surprisingly, it has now also been found that other fragments of the protein react with auto-antibodies against the U<sub>2</sub>-snRNP-B<sup>+</sup> protein and the Sm protein, two other snRNP proteins. The presence of antibodies against the Sm protein form an indication that the patient is developing SLE, whilst the occurrence of antibodies against the U<sub>1</sub>-snRNP-A and U<sub>2</sub>-snRNP-B<sup>+</sup> proteins provides an indication of MCTD.

50 The fragments of the protein of formula 1 which react with antibodies against U<sub>1</sub>-snRNP-A, U<sub>2</sub>-snRNP-B<sup>+</sup> and Sm protein have the amino acid sequences shown in formula 2, 3, and 4 respectively. The invention also relates to smaller fragments of these amino acid sequences mentioned provided these smaller fragments are still immunochemically reactive, such as the amino acid sequence of formula 2a (being a smaller fragment of the peptide of formula 2) and the amino acid sequence of formula 3a (being a smaller fragment of the peptide of formula 3). The invention further relates to peptides which contain such

fragments.

The preparation of these immunochemically reactive protein fragments according to the invention follows known routes, such as chemical synthesis or recombinant DNA techniques.

The protein, the fragments and the peptide according to the invention can be used for the detection of the auto-antibodies mentioned using diagnostic methods known per se for the determination of antibodies by means of antigens reactive with them.

Both homogeneous and heterogeneous diagnostic tests are suitable for this purpose. Use can thus be made of sandwich type tests or of an agglutination test in which, if desired, an inhibition or competition reaction is used.

Suitable tests are those in which a solid phase is used, such as the inner wall of a microassay well, a tube or capillary, a membrane, filter, test strip or the surface of a particle to which an antibody or antigen is bound. For the detection, use is thus made of an antigen or antibody which is provided with a label, such as a radio-active isotope, a dyestuff, metal sol such as a gold sol, an enzyme, or another known label. Methods for the preparation of labelled antigens or antibodies are generally known.

Moreover, the protein, fragments thereof, and peptides which contain such fragments, can be used in suitable pharmaceutical dosage forms against auto-immune diseases. For example, purified U<sub>1</sub>snRNP-A antigen can be injected intravenously into a patient who is producing antibodies against this antigen. One should administer to a human preferably 1-1000 nanomoles of said A-antigen.

Administration of such a preparation via the circulatory system of a patient with an auto-immune disease results in an antigen-antibody complex formation which can decrease further attack on the abovementioned tissues. The diagnostic methods mentioned can be used to determine the precise quantity of auto-antibodies circulating in the blood. The quantity of the preparation to be administered is determined by the result of said test. The invention is illustrated by reference to the following Examples.

#### Example I

##### Nucleotide sequence and inferred amino acid sequence of the A protein

Starting from the material lodged with the Central Office for Mould Cultures in Baarn, The Netherlands under number CBS 817.87, the nucleotide sequence of the cDNA inserts has been determined using the so-called dideoxy method described by Sanger et al. (PNAS, 74, 5463-5467, 1977). This nucleotide sequence is shown in formula 5. The initiation codon ATG is located at nucleotide position 126.

The stop codon TAG is located at nucleotide position 972, followed by a non-coding region of 223 nucleotides. The inferred amino acid sequence of 282 amino acids for the A protein (formula 1) gives a molecular weight of 31.2 kd, which is in good agreement with the observed molecular weight of 32 kd for the A protein analyzed using a SDS-polyacrylamide gel.

#### Example II

##### Mapping of an antigenic determinant of the A protein

The amino acid sequence of the A protein inferred from the nucleotide sequence as given, has an interesting distribution of charged and aromatic amino acids. These can be assigned to two regions, separated by a segment with a very high proline content (amino acid 140 to 206 in figure 1). Most of the charged and aromatic amino acids are located on the N-terminal part of the protein. The amino acid sequence of the A protein, according to Formula 1, can be split into two parts, namely amino acid 1 to 171 and amino acid 172 to 282. The DNA sequences coding for these sequences, are each placed separately in a suitable expression vector with recombinant DNA techniques known per se. The proteins synthesized by this expression vector are then brought into contact with patient serum containing auto-antibodies via an immunoblotting experiment. Patient serum containing anti-Sm-antibodies reacts specifically with the N-terminal part (amino acid 1 to 171) of the A protein. In this N-terminal part there is a cluster of eleven charged amino acids which represent a hydrophilic section of the A protein (amino acid 103 to 112 in

formula 1). Hydrophilicity analysis using the Hopp and Woods method supports this site determination. This sequence Glu - Arg - Asp - Arg - Lys - Arg - Glu - Lys - Arg - Lys in the A protein thus forms an epitope which is recognized by patient serum which contains anti-Sm antibodies. Moreover, another unexpected aspect relating to the amino acid sequence of the A protein emerges from this immunoblotting experiment. Both patient serum containing anti-B-antibodies and patient serum containing anti-A-antibodies react in an immunoblotting experiment with the C-terminal part of the A protein (amino acid 172 to 282 in formula 1). If the inferred amino acid sequence of the A protein is laid in a line with the known amino acid sequence of B protein, on the grounds of this overlap it can then be determined by exclusion that the specific epitope responsible for the antigen character with respect to anti-A-antibodies is located in the A protein in the region of amino acid 172 to 207 according to formula 1; this sequence represents a strongly hydrophilic area, and hydrophilicity analysis using the Hopp and Woods method supports this epitope mapping. This also establishes that the epitope which cross-reacts with anti-B-antibody is located in the region of amino acid 207 to 282 in formula 1.

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### Example III

#### 20 Enzyme Immuno Assay (EIA)

An EIA was set up in which was tested 480 sera from patients with connective tissue diseases. Simultaneously, the same serum samples were tested on immunoblots with eukaryotic nuclear proteins as antigen. The intensity of the reaction on these blots was scored on an arbitrary scale ranging from "very weak" to "strong". When sera were thus grouped on basis of increasing titer of antibodies against eukaryotic A or B proteins, the signals obtained in the EIA appeared to correlate perfectly with the blotting results; for the two antigens tested, an increased blotting signal corresponded with an increased EIA reading. When patients were subsequently grouped on basis of their diagnosis, it appeared that this simple and sensitive EIA allowed to distinguish between patients with definite SLE and patients with MCTD. Especially the presence of antibodies against the A protein appeared to be diagnostic for MCTD, since they do not, or in very low titers, occur in definite SLE patients.

#### Claims

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1. A protein with the structure as shown in formula 1, which protein is substantially free from other natural material, and fragments thereof which are immunochemically reactive and peptides which contain such fragments.
2. Amino acid sequences according to claim 1 of the formula 2 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.
3. Amino acid sequences according to claim 1 of the formula 3 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.
4. Amino acid sequences according to claim 1 of the formula 4 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.
- 45 5. Amino acid sequence according to claim 1 or 2 of the formula 2a.
6. Amino acid sequence according to claim 1 or 3 of the formula 3a.
7. DNA coding for the fragments, the peptides, and the protein according to claim 1.
8. Vector which contains DNA according to claim 7.
9. Host which contains the vector according to Claim 8.
- 50 10. Method for the preparation of the protein according to Claim 1, and of fragments thereof which are immunochemically reactive and of peptides which contain such fragments, characterized in that the protein, the fragments, and the peptides are prepared according to a method known per se using peptide synthesis techniques and/or recombinant DNA techniques.
11. Method for the determination of an auto-antibody in a sample, characterized in that, use is made of the protein according to Claim 1 or a fragment thereof which is immunochemically reactive or a peptide which contains such a fragment in which at least one part of the protein, fragment, or peptide is provided with a label.
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12. Test kit for the determination of an auto-antibody with the characteristic that the test kit, along with the usual reagents for these determinations, also contains a protein according to Claim 1 or a fragment thereof which is immunochemically reactive or a peptide which contains such a fragment in which at least on part of the protein fragment or peptide is provided with a label.

13. Pharmaceutical preparation containing, along with the usual additions, the protein according to Claim 1 as active component, or a fragment thereof which is immunochemically reactive, or a peptide which contains such a fragment.

# Formula 1

1 Met Ala Val Pro Glu Thr Arg Pro Asn His Thr Ile Tyr Ile Asn Asn Leu Asn Glu Lys Ile Lys Lys Asp Glu Leu Lys Lys Ser Leu 20  
 31 Tyr Ala Ile Phe Ser Glu Phe Gly Glu Ile Leu Asp Ile Leu Val Ser Arg Ser Leu Lys Met Arg Glu Ala Phe Val Ile Phe Lys 40  
 61 Glu Val Ser Ser Ala Thr Asn Ala Leu Arg Ser Met Glu Gly Phe Pro Phe Tyr Asp Lys Pro Met Arg Ile Glu Tyr Ala Lys Thr Asp 60  
 91 Ser Asp Ile Ile Ala Lys Met Lys Gly Thr Phe Val Glu Arg Asp Arg Lys Glu Lys Arg Lys Pro Lys Ser Glu Glu Thr Pro Ala 80  
 121 Thr Lys Lys Ala Val Glu Gly Gly Ala Thr Pro Val Val Glu Ala Val Glu Gly Pro Val Pro Glu Met Pro Pro Met Thr Glu Ala 100  
 151 Pro Arg Ile Met His Met Pro Gly Glu Pro Pro Tyr Met Pro Pro Gly Met Ile Pro Pro Gly Leu Ala Pro Gly Glu Ile 120  
 181 Pro Pro Gly Ala Met Pro Pro Glu Glu Leu Met Pro Gly Glu Met Pro Pro Ala Glu Pro Leu Ser Glu Asn Pro Pro Asn His Ile Leu 140  
 211 Phe Leu Thr Asn Leu Pro Glu Glu Thr Asn Glu Leu Met Leu Ser Met Leu Phe Asn Glu Phe Pro Gly Phe Lys Glu Val Arg Leu Val 160  
 241 Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Glu Ala Glu Ala Arg Asp Ala Leu Glu Gly Phe Lys Ile Thr 180  
 271 Glu Asn Asn Ala Met Lys Ile Ser Phe Ala Lys Lys

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Pro Pru Ciy Low Ala Pro Ciy Cila Ilo Pru Pru Ciy Ala Pru

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The Law The Law Pro Ciu Ciu The Adu Ciu Low Not Low Ser Not Low Png Das Lin Png Pri. Cig Png Lys Ciu Val Arg Low Val

Pro C1y Arg M1a Asp M1a Pro Val C1a Pro Asp Asn C1u Val C1a C1y M1a Als Arg Asp Als Leu C1a C1y Pro Lys Ile Phe

UIC 400 400 410 420 430 440 450 460 470 480 490

**Pro Ciy Pno Lys Ciu Vat Arg Leu Vat Pro Ciy Arg Mts Asp Ile Ala Pno Met Ciu Pno Asp Asn Ciu Val Ciu Ala Ciy Ala Ala Arg Asp Ala Leu Cila**

017 844 061 019 844 061 844 061 844 019

Formula 5

100 200 300 400 500 600 700 800 900 1000  
 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000  
 2100 2200 2300 2400 2500 2600 2700 2800 2900 3000  
 3100 3200 3300 3400 3500 3600 3700 3800 3900 4000  
 4100 4200 4300 4400 4500 4600 4700 4800 4900 5000  
 5100 5200 5300 5400 5500 5600 5700 5800 5900 6000  
 6100 6200 6300 6400 6500 6600 6700 6800 6900 7000  
 7100 7200 7300 7400 7500 7600 7700 7800 7900 8000  
 8100 8200 8300 8400 8500 8600 8700 8800 8900 9000  
 9100 9200 9300 9400 9500 9600 9700 9800 9900 10000



Formula 1

1 Met Ala Val Pro Glu Thr Arg Pro Asn His Thr Ile Tyr Ile Asn Asn Leu Asn Glu Lys Ile Lys Asp Glu Leu Lys Lys Ser Leu 90  
 31 Tyr Ala Ile Phe Ser Glu Phe Glu Ile Leu Asp Ile Leu Val Ser Arg Ser Leu Lys Met Arg Gly Glu Ala Phe Val Ile Phe Lys 60  
 61 Glu Val Ser Ser Ala Thr Asn Ala Leu Arg Ser Met Glu Gly Phe Pro Phe Tyr Asp Lys Pro Met Arg Ile Glu Tyr Ala Lys Thr Asp 90  
 91 Ser Asp Ile Ile Ala Lys Met Lys Gly Thr Phe Val Glu Arg Asp Arg Lys Arg Glu Lys Arg Lys Pro Lys Ser Glu Glu Thr Pro Ala 120  
 121 Thr Lys Lys Ala Val Glu Glu Gly Glu Ala Thr Pro Val Val Glu Val Glu Glu Pro Val Pro Gly Met Pro Pro Met Thr Glu Ala 150  
 151 Pro Arg Ile Met His Met Pro Gly Glu Pro Pro Tyr Met Pro Pro Gly Met Ile Pro Pro Gly Leu Ala Pro Gly Glu Ile 180  
 181 Pro Pro Gly Ala Met Pro Pro Glu Glu Leu Met Pro Gly Glu Met Pro Pro Ala Glu Pro Leu Ser Glu Asn Pro Pro Asn His Ile Leu 210  
 211 Phe Leu Thr Asn Leu Pro Glu Glu Thr Asn Glu Leu Met Leu Ser Met Leu Phe Asn Glu Phe Pro Gly Phe Lys Glu Val Arg Leu Val 240  
 261 Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Glu Ala Glu Ala Arg Asp Ala Leu Glu Gly Phe Lys Ile Thr 270  
 271 Glu Asn Asn Ala Met Lys Ile Ser Phe Ala Lys Lys

Formula 1

Pro Pro Gly Leu 10 Pro Gly Val Ile Pro Pro Gly Ala Met Pro Pro Gly Val Leu Met Pro Gly Val Met Pro Pro Ala Val Pro Leu Ser Glu Val Pro Pro Ala

Formula 2a

Pro Pro Gly Leu Ala Pro Gly Val Ile Pro Pro Gly Ala Met

Formula 3

Asn His Ile Leu

Pro Leu Thr Asn Leu Pro Gly Val Thr Asn Gly Leu Met Leu Ser Met Leu Pro Asn Val Pro Gly Thr Lys Glu Val Arg Leu Val

Pro Gly Arg His Asp Ile Ala Pro Val Glu Thr Asp Asn Gly Val Glu Ala Gly Ala Ala Arg Asp Ala Leu Gly Gly Thr Lys Ile Thr

Glu Asn Asn Ala Met Lys Ile Ser Pro Ala Lys Lys

Formula 3a

Pro Gly Thr Lys Glu Val Arg Leu Val Pro Gly Arg His Asp Ile Ala Pro Val Glu Thr Asp Asn Gly Val Glu Ala Gly Ala Ala Arg Asp Ala Leu Gly

Formula 4

Glu Arg Asp Arg Lys Arg Glu Lys Arg Lys

**Formula 5**

[illegible]



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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 83, November 1986, pages 8689-8693, The National Academy of Sciences, Washington, D.C., US; R. REUTER et al.: "Immunization of mice with purified U1 small nuclear ribonucleoprotein (RNP) induces a pattern of antibody specificities characteristic of the anti-Sm and anti-RNP autoimmune response of patients with lupus erythematosus, as measured by monoclonal antibodies" * Whole article *	1-13	C 07 K 13/00 C 12 N 15/00 G 01 N 33/68 A 61 K 37/02 G 01 N 33/564
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, no. 9, 10th May 1984, pages 5907-5914, The American Society of Biological Chemists, Inc., US; I. PETTERSSON et al.: "The structure of mammalian small nuclear ribonucleoproteins" * Whole article *	1-13	
A	THE EMBO JOURNAL, vol. 5, no. 13, 1986, pages 3509-3516, IRL Press Ltd, Oxford, GB; P. BRINGMANN et al.: "Purification of the individual snRNPs U1, U2, U5 and U4/U6 from hela cells and characterization of their protein constituents" * Pages 3509-3516; page 3510, figure 2; page 3513, figure 6 *	1-13	C 07 K C 12 N G 01 N A 61 K C 12 P
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 27-01-1989	Examiner HERMANN R.R.W.
CATEGORY OF CITED DOCUMENTS			
X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document		T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons *: member of the same patent family, corresponding document	



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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	THE EMBO JOURNAL, vol. 2, no. 7, 1983, pages 1129-1135, IRL Press Ltd, Oxford, GB; P. BRINGMANN et al.: "Purification of snRNPs U1, U2, U4, U5 and U6 with 2,2,7-trimethylguanosine-specific antibody and definition of their constituent proteins reacting with anti-Sm and anti-(U1)RNP antisera" * Whole article * -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			

Place of search  
THE HAGUE

Date of completion of the search  
27-01-1989

Examiner  
HERMANN R.R.W.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone  
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